AD 655170

JOURNAL OF VIROLOGY, June 1967, p. 453-459
Vol. 1, No. 3

Properties of Venezuelan Equine Encephalomyelitis Virus Accompanying Attenuation In Vitro

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Received for publication 30 December 1966

Virus obtained during serial plaque passage of the virulent parent egg seed (PES) of the Trinidad strain of Venezuelan equine encephalomyelitis (VEE) virus produced only large plaques during either 3 serial plaque passages in chick fibroblasts or 10 plaque passages in L cells, and was lethal for mice by the intraperitoneal route. Virus showing these characteristics was designated the stable large-plaque (Ls) type. In contrast, virus obtained during serial plaque passage of the attenuated 9t strain in chick fibroblasts formed only very small plaques and was not lethal for mice by the intraperitoneal route. Virus showing these properties was designated the stable small-plaque (Ss) type. Under other passage conditions, however, large-plaque virus that yielded about 90% large and 10% small plaques was obtained; this virus was designated the unstable large or Lu type because it differed from the Ls type, which yielded only large plaques. The Lu type continued to yield the same ratio of large to small plaques for several plaque-to-plaque passages. In addition, small-plaque virus that yielded both large and small plaques and that showed a reduced capability to infect mice was also recovered. This virus was designated the unstable small or Su type because it differed from the Ss type in its higher level of virulence and in its plaque-forming properties. Thus, based upon the properties of virulence for mice and plaque size, four viral types could be discerned. The evidence suggests that serial passage in cell culture imposed environmental pressures that sequentially selected the following viral types: Ls, Lu, Su, and Ss.

Several Venezuelan equine encephalomyelitis (VEE) virus variants exhibit a marked loss in virulence for common laboratory animals after passage in cell cultures (2, 10, 14, 19, 20). Perhaps the most significant of these is the attenuated strain of Berge et al. (2), which appears to be suitable as a live vaccine for humans (17). In our laboratory, studies with a VEE virus variant show that the loss in virulence of the virus is attended by a loss in ability to form large plaques (9); similar findings with another VEE virus variant were reported by Mussgay and Suarez (20). Brown (3) reported that a high-passage preparation of the avirulent strain isolated by Berge and his colleagues apparently produces only small plaques. Heydrick et al. (11) showed that, during 10 serial passages of VEE virus in L cells or chick fibroblasts, plaque size begins to decrease as attentuation becomes demonstrable. Detailed information, however, concerning mutational events that may occur during the conversion to small-plaque attenuated VEE virus strains is lacking. In this report, an attempt is made to recognize and describe some of the events that occur during the attenuation of our strains of VEE virus by passage in vitro. Viral populations arising under various conditions of selection and passage are studied and characterized.

MATERIALS AND METHODS

Virus strains. The parent egg seed (PES) of the Trinidad strain of VEE virus, described elsewhere (10), was prepared after 13 passages in embryonated eggs.

The attenuated virus (9t) strain was obtained from an L-cell monolayer culture that had become chronically infected after inoculation of the PES strain The virus was harvested 8 months after initial infection and underwent a number of serial passages in fresh L cells. The virus from the ninth such serial passage was designated the 9t strain (10).

Cell cultures for growth and plaque assay. Propagation and plaque assays of virus were performed in chick fibroblasts and, when indicated, in L-cell monolayers. Chick fibroblasts were prepared by a modification of the procedure described by Dulbecco (7). They were grown in medium 199 supplemented with 20% horse serum plus 0.1 mg of streptomycin and 100 units of penicillin per ml. Confluent cell sheets were routinely obtained in 30-mm plastic plates after 48 hr of incubation at 37 C in an atmosphere of 5%

carbon dioxide. L-cell cultures were obtained from stock cultures at these laboratories and grown in a similar manner.

Virus suspensions to be assayed were routinely diluted in 0.1% yeast extract-0.1% Proteose Peptone No. 3 (Difco) in Hanks balanced salt solution (BSS), *p*H 7.4, and appropriate concentrations were inoculated directly onto the cell sheets to provide counts of 5 to 50 plaques, ranging over two 10-fold dilutions. After a 30-min adsorption period, the excess inoculum was removed, the cell sheet was washed once with the diluent, and 5 ml of agar overlay was added. The overlay consisted of 0.45% lactalbumin hydrolysate, 0.09% yeast extract, 1.1% agar (Difco), and 0.5% gelatin. Infected monolayers were incubated at 37 C with 5% CO_2 for 72 hr and were stained by adding a second agar overlay containing 1:10,000 neutral red. Plaques were visible within 3 hr after staining.

Virus from individual plaques was isolated with a capillary pipette that removed agar, virus, and a portion of the cell sheet within the plaque. This was dispersed in 2.5 ml of yeast extract-peptone diluent, containing 50% horse serum, and was considered as undiluted plaque-virus. Generally, such isolates were stored at −55 C before use. Virus was then inoculated directly onto fresh chick fibroblasts or L cells for plaque assay and for tests for virulence in mice. In some instances, because of the small quantity of virus present in some plaques, isolates were grown in fluid cultures of chick fibroblasts for 48 to 72 hr to increase the virus concentration prior to the plaque assays and inoculations in mice. Titers of such viral suspensions were 10^6 to 10^7 MICLD_{50} per ml.

Virus titrations. Virus samples were titrated intracerebrally and intraperitoneally in 12- to 14-g Swiss white mice; the titers are expressed as MICLD_{50} and MIPLD_{50} per milliliter, respectively. In most instances, particularly with virus from single plaques, titration was not necessary, and an appraisal of virulence was obtained by injecting mice with undiluted virus or 1:10 dilutions of virus, or both. Mice that survived injections with the plaque material were challenged by the intraperitoneal route after 21 days with a multiple lethal dose ($10^{3.5}$ to $10^{4.5}$ MICLD_{50}) of the PES strain. Mice that were immune to challenge were considered as having been subclinically infected as a result of the initial injection.

RESULTS

Characterization of the virulent parent (PES) and attenuated (9t) VEE virus strains. Plaques ordinarily formed by the PES strain during first passage in chick fibroblasts ranged from 3 to 6 mm in diameter; the majority were 4 mm. In all of our experiments, in which many hundreds of plaques were examined, diameters smaller than 3 mm were rarely observed. Although the PES strain possesses limited virulence in monkeys, eliciting chiefly a febrile, nonlethal response, this strain is uniformly lethal in mice and other laboratory animals when injected intraperitoneally. Results of tests for virulence and plaque size, performed with virus obtained during the second and third plaque passages, were the same as those during the first passage. During the fourth, sixth, and ninth serial passage of large-plaque virus in chick fibroblasts, however, both large and small (2 mm) plaques were found; when present, the latter amounted to 8 to 10% of the total plaque number. Results of tests during 10 serial passages of large-plaque virus in L cells were similar to those found during three serial passages in chick fibroblasts; small-plaque virus was rarely observed.

Virus from the PES strain that formed only large plaques during either 3 passages in chick fibroblasts or 10 passages in L cells appeared homogeneous and genetically stable. Such virus was designated as a stable, large-plaque (Ls) type. Large-plaque virus that yielded mixtures of both large and small plaques beyond the third serial plaque passage in chick fibroblasts was designated as an unstable, large-plaque (Lu) type. Virus isolated from the large plaques in either cell type was lethal for mice by the intraperitoneal route. A more detailed characterization of Lu plaque types isolated from the PES strain is presented in a later section of this report.

In contrast to results with the PES strain, the 9t strain formed small plaques (0.5 to 1.5 mm) in chick fibroblasts. Except for a few deaths found occasionally with undiluted virus, the strain was nonlethal for mice by the intraperitoneal route.

The virus isolated from single, small plaques formed only small plaques containing attenuated virus during either 4 plaque passages in chick fibroblasts or 10 passages in L cells. For this reason, virus of this type was considered, by definition, to be relatively homogeneous and genetically stable. It was designated as a stable small-plaque (Ss) type.

Derivation of unstable large-plaque (Lu) types from the 9t strain. In one experiment, the virus from six small plaques was pooled after the fourth serial passage of small-plaque virus from the 9t strain. This preparation yielded plaques of which 2 (4%) were 0.5 and 1.5 mm and 44 (96%) were 2 to 3 mm in diameter. On the same plate, one 7-mm plaque was found. The emergence of plaques of the 2- to 3-mm range from small-plaque virus of the attenuated strain possibly represented a rare instance in which plaques were 1 to 2 mm larger than those usually observed. This observation might have resulted from undetermined environmental changes that temporarily influenced plaque size only. Another explanation for the appearance of the 2- to 3-mm plaques is that they might have been intermediate between the large type usually produced by the parent (PES) strain and the small attenu-

ated (9t) type with respect to size and perhaps to virulence. In the absence of immediately suitable evidence to the contrary, and for purposes of classification, these plaques were tentatively regarded as small plaques. The 7-mm plaque, however, contained particles that were lethal for mice when injected intraperitoneally. Virus isolated from this plaque was selected for further study.

The data in Fig. 1 show that the virus isolated from the large, 7-mm plaque formed 87% large and 13% small plaques. This virus resembled the stable, large plaque (Ls) virus found in the PES strain, because it formed large plaques and was lethal for mice. It differed from the Ls type, however, because it was unstable upon passage and produced a high percentage of small plaques. For this reason, this virus was considered as another example of the unstable large-plaque (Lu) type. The results of the passage of virus from two 5-mm plaques that came from the 7-mm plaque also are represented in Fig. 1. The virus from one of these, designated plaque 5a, produced 94% large and 6% small plaques (shown on line A). Deliberate selection and passage of the virus from large plaques continued to result in the formation of approximately 90% large and 10% small plaques. Virus recovered from the large plaques was always virulent for mice.

The results suggest that the virus from plaque 5a and its progeny was genetically unstable and indistinguishable from that of the parent 7-mm plaque. Large-plaque isolates from this line, therefore, continued to warrant the Lu designation.

In contrast, the virus isolated from plaque 5b immediately produced 100% small plaques during the same experiment in which the results from the passage of virus in plaque 5a were obtained. Figure 1 (line B) shows that two passages of this small-plaque virus resulted in the continued formation of 100% small plaques. A further passage of virus from a pool of four plaques again resulted in 100% small plaques. Comparable results were obtained for eight additional passages, and mice that were injected with the avirulent small-plaque virus were resistant to challenge with the lethal PES strain.

Figure 1 (top of line B) also shows that the virus obtained from a 2-mm plaque, and passed at the same time as the virus described above, contained only virus that yielded small (1-mm) plaques. Virus from a pool of five small plaques was serially passed twice in chick fibroblast fluid cultures to provide an opportunity for large-

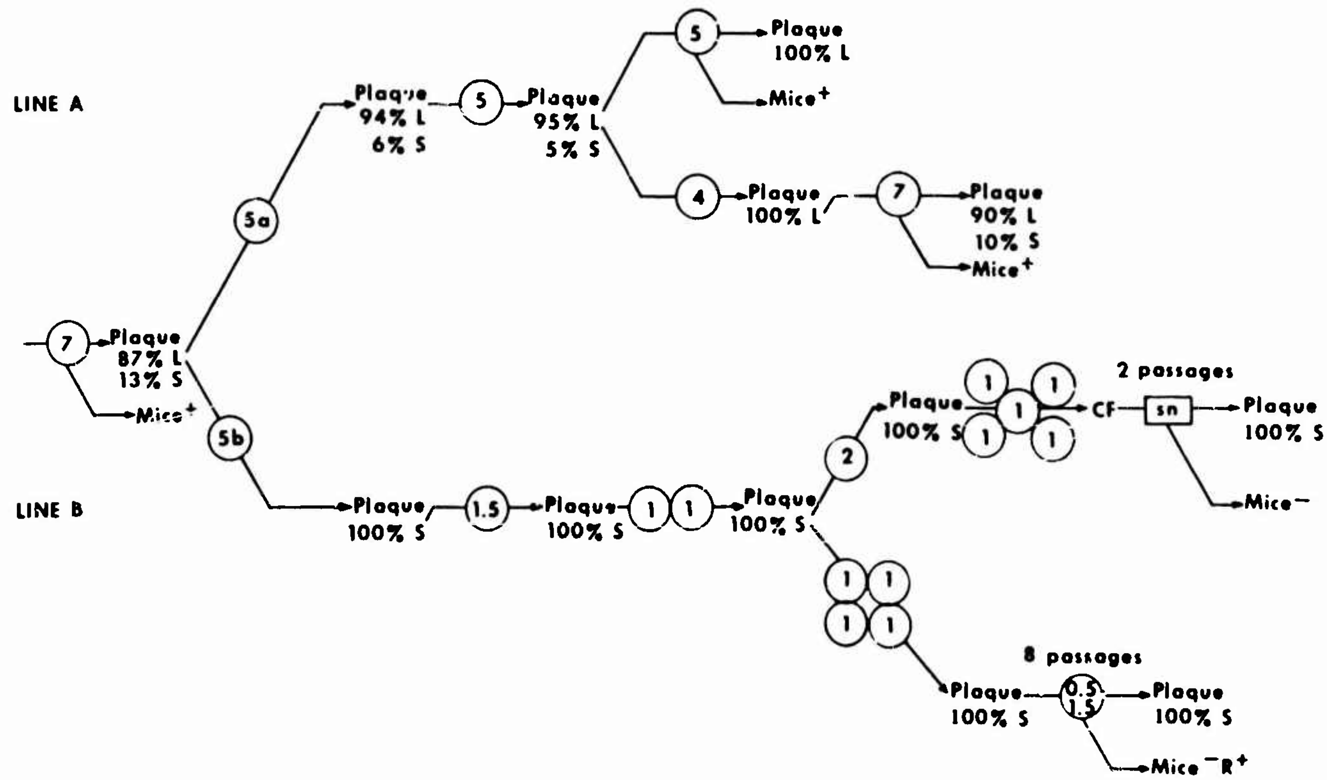

FIG. 1. *Comparison of viral progeny from two large phenotypically similar plaque types isolated from a single large unstable plaque type. Numbers enclosed in circles represent passage of virus from the indicated plaque size; L = large plaque (>2 mm in diameter); S = small plaque (<2 mm in diameter); CF = chick fibroblast fluid culture; sn = infected CF supernatant fluid; mice+ = virus lethal by the intraperitoneal route; mice R+ = virus nonlethal by the intraperitoneal route and mice resistant to intraperitoneal challenge with a multiple lethal dose of PES virus.*

plaque, virulent virus to emerge. Once again, virus in the culture supernatant fluid produced only small plaques and was not lethal for mice.

The results obtained with the virus from plaque 5b suggest that it represented an Lu type that may have contained a very small percentage of large-plaque virus. Passage of the 5b plaque isolate gave rise to a stable, small-plaque (Ss) type. Additional examples of phenomena of this type are discussed in the next section.

Derivation of unstable small-plaque (Su) and stable small-plaque (Ss) types from unstable large-plaque (Lu) types. Results of attempts to examine more critically the progeny obtained after passage of virus from Lu plaque types are presented below and in Fig. 2. These data show that virus from Lu plaques gave rise to an unstable small (Su) variety that, in turn, formed stable small-plaque (Ss) types.

As one example of this, virus from plaque 5a was serially passed three times in chick fibroblast fluid cultures to provide suitable quantities of working material. The results are shown in Fig. 2, line A. Viral material from the third serial passage formed 63% large and 37% small plaques; it was lethal for two of six mice and induced paralytic illness in two others that eventually recovered. Continuing along line A, virus isolated from a small plaque (1.5 mm) formed 33% large and 67% small plaques. Because the virus from this plaque contained significant quantities of both large- and small-plaque-forming particles, it was considered to be an unstable, small-plaque (Su) type. After the virus was passed in chick fibroblast fluid cultures, however, it produced 100% plaques of the Ss type and was nonlethal for mice. The infectivity of the Ss virus in mice was demonstrated by the resistance of the mice to a challenge with a multiple lethal dose of the PES strain.

Under another set of circumstances (Fig. 2, line B), the virus from plaque 5a was serially passed only twice in chick fibroblast fluid cultures. Virus obtained 48 hr postinoculation during the second passage produced only large plaques. Virus obtained at 72 hr from the same cultures (line C), however, produced 82% large and 18% small plaques; this virus was lethal for mice. The opportunity for additional cycles of viral growth from 48 to 72 hr in the chick fibroblasts apparently led to the appearance of small quantities of small-plaque virus. Continuing along line C, virus from a 1.5-mm plaque, representative of the small plaques on the plate, was

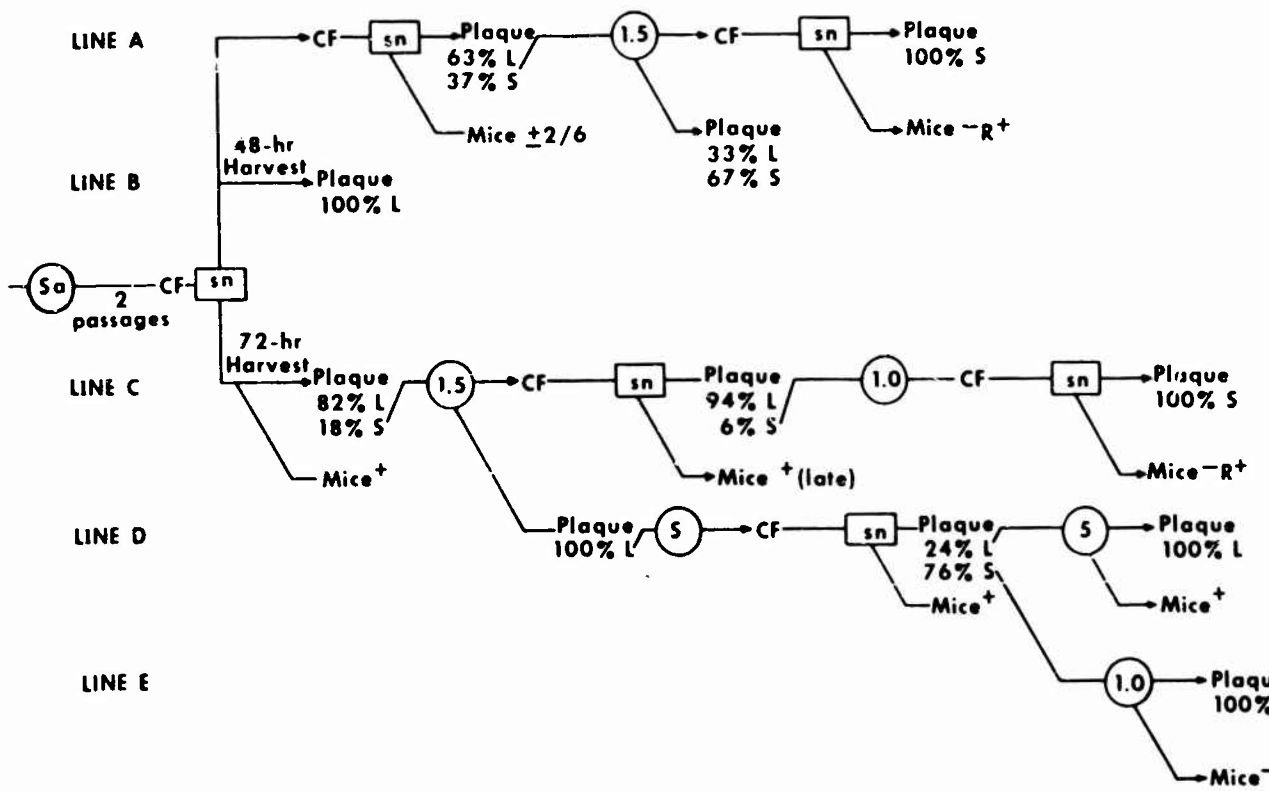

FIG. 2. *Derivation of large unstable, small unstable, and small stable plaque types from a large unstable plaque type. The encircled 5a indicates virus from the same plaque shown in Fig. 1; other encircled numbers represent passage of virus from the indicated plaque size; L = large plaque (>2 mm in diameter); S = small plaque (<2 mm in diameter); CF = chick fibroblast fluid culture; sn = infected CF supernatant fluid; mice$^+$ = virus lethal by the intraperitoneal route; mice$^-$R$^+$ = virus nonlethal by the intraperitoneal route and mice resistant to intraperitoneal challenge with a multiple lethal dose of PES virus; mice ±2/6 = virus lethal for two of six mice and two paralyzed mice recovered; mice$^+$ (late) = mean day of death delayed 48 hr.*

passed in chick fibroblast fluid cultures to increase the titer and then was subjected to a plaque test.

After passage of the 1.5-mm plaque virus in fluid culture (line C), the harvested material produced 94% large plaques and 6% small plaques, and was lethal for mice. Thus, the virus from this (1.5-mm) plaque was not typical of stable, small-plaque virus of the Ss type because it contained virus capable of forming both large and small plaques and producing lethal infections in mice by the intraperitoneal route. It is of interest that mice that succumbed to this virus did so 2 to 4 days beyond the usual course of the disease. For these reasons, this atypical, small-plaque virus from the 1.5-mm plaque was considered to be another example of the unstable small (Su) type. From the resultant 6% small plaques, 1-mm plaque virus was isolated and grown in chick fibroblast fluid cultures. Supernatant fluids from these cultures contained 100% small-plaque virus. This virus appeared to be similar, if not identical, to virus of the Ss type. That the mice were sublethally infected by Ss type virus was shown by the fact that they resisted a challenge with a multiple lethal dose of the PES strain.

Returning to the information in Fig. 2, line D, it can be seen that virus obtained directly from the 1.5-mm Su plaque (shown on line C), and passed at the same time as the small plaques discussed above, produced only large plaques. Virus from the large plaques appeared to be of the Lu type. As shown on line D, large plaques were encountered in the minority (e.g., 24% large versus 76% small) if the large-plaque virus had an intervening passage in chick fibroblast fluid cultures prior to being plaque-tested. These data support the previous observations that the passage of virus of the Lu type in chick fibroblast fluid cultures, allowing multiple cycles of replication, appeared to favor the selection of small-plaque virus. The final results, on line E (far right side of Fig. 2), indicate that small-plaque virus of the Ss type also could be recovered from supernatant fluids of chick fibroblast cultures infected with a 5-mm, Lu plaque type. Typically, this Ss virus formed only small plaques and was nonlethal for mice; animals injected with this virus resisted a later challenge with a multiple dose of the PES strain.

Derivation of attenuated, stable small-plaque (Ss) types from the virulent, stable large-plaque (Ls) type. The foregoing data show that passage of virus in chick fibroblasts provided conditions that were selective for stable small-plaque, attenuated virus. Thus far, however, Ss virus was demonstrated as having been derived only from large plaques of the Lu type in the 9t strain. This raised the question as to whether the Ss type could be derived from the Ls type, of which the virulent PES strain is almost entirely composed.

It has already been mentioned that three serial passages of PES strain virus from large plaques resulted in yields of similar plaque types. It was found, however, that, by taking advantage of the selective environmental conditions apparently existing during the subsequent serial plaque passage of virus in chick fibroblasts, certain small-plaque-forming mutants could be recognized and isolated. As an example of this, from among the large plaques formed by the PES virus material, the virus from four 3-mm plaques was pooled. Upon passage of this pool, the majority of plaques was again found to be large (3 to 4 mm), but several were as small as 2 mm. This suggested that the initial step, the conversion of virus from the Ls to that of the Lu type, had occurred. Passage of one of the 2-mm plaques resulted in progeny the majority of which produced plaques approximately 2 mm in size, and several produced plaques as small as 0.5 mm. This suggested the occurrence of a phenomenon similar to that discussed in previous sections, in which the Lu type gave rise to virus of the Su type.

From among the small (<2 mm) plaques, virus from a single 1-mm plaque was passed further, resulting in plaques ranging between 0.5 and 2.0 mm in diameter. This last procedure was repeated to make a total of 10 serial passages from the PES strain starting material. Virus that was harvested from 1-mm plaques had become nonlethal for mice by the intraperitoneal route at this passage level. Previous to the tenth passage, some evidence of virulence for mice by the intraperitoneal route had persisted. Results during the eleventh and twelfth serial passages demonstrated that stable small-plaque (0.5 to 1 mm), attenuated virus had arisen after 10 serial passages of virus from the stable large-plaque, virulent PES strain.

DISCUSSION

The serial passage of various isolates of VEE virus in chick fibroblasts disclosed the presence of environmental pressures that, in the main, selected for small-plaque, attenuated particles. These viral types could be recognized by their reduced ability to form large plaques under agar and cause lethal illness in mice by the intraperitoneal route. This provides additional support for related observations on plaque size and virulence that have been made previously with VEE virus (3, 9, 10, 20) and other viruses, such as poliovirus (23), vesicular exanthema virus of swine (16), coxsackievirus (12), and mengovirus (6). It is interesting to note, however, that attenuation has

not been found to be associated with small-plaque isolates of Sindbis virus (8) or Rift Valley fever virus (Boyle, *personal communication*).

Less efficient counter-pressures appeared to cause a sporadic recurrence of small numbers of large-plaque virulent particle types. This was disclosed initially during our examination of a pool of virus from six small plaques that were obtained after three serial plaque passages of virus from the attenuated 9t strain. Although the large-plaque back-mutants encountered during our experiments superficially resembled the large-plaque virus of the unattenuated PES strain, the virus contained in the large plaque was, in fact, different. This virus consistently yielded relatively uniform mixtures of large and small plaques upon passage under agar, in a manner similar to that shown by western equine encephalomyelitis virus (13), whereas large-plaque virus isolated directly from the PES strain yielded large plaques only. Because of this difference, virus from large plaques that yielded both large and small plaques was designated the unstable large or Lu type, and the virus from the PES strain was designated the stable large or Ls type. It is of further interest that the small plaques obtained from passage of the large-plaque back-mutant contained small-plaque virus that resembled the attenuated strain in plaque size. This similarity in plaque size also proved to be superficial, because the small-plaque virus (Su) isolated from the Lu type large-plaque almost always showed a higher level of virulence than that of the Ss virus from the attenuated strain. In addition, the unstable small (Su) types almost always yielded mixtures of large and small plaques upon passage. Virus from the Ss type yielded small plaques only. Continued passage of virus of the Su type resulted in the recovery of Ss-type virus typical of that found in the 9t strain.

Thus, based upon the properties of virulence for mice and plaque size, four viral types could be discerned. When the data on the occurrence of these plaques were studied in detail, these types did not appear to emerge at random but rather in a predictable order. The evidence indicates that serial passage in vitro imposed environmental pressures that sequentially selected the following viral types: Ls, Lu, Su, and Ss. The data also raise a question of the possible existence of other as yet undiscerned VEE viral types. Evidence suggesting that stepwise changes might be operational in the selection of other viral mutants has recently been reviewed by Walen et al. (25). These investigators have indicated that several mutations might be necessary during conversion of the diminutive to large-plaque types with vesicular exanthema virus of swine. On the other hand, the alteration from the minute to the large-plaque type as a single mutational event is also mentioned as a possibility. An event similar to this may have occurred during one of our previous studies (Hearn and Soper, Bacteriol. Proc., p. 135, 1962), in which we found that, despite the loss in virulence of our small-plaque-forming attenuated 9t strain for a variety of laboratory animals, the hamster remained uniformly susceptible to lethal infection with high dilutions of this virus and that virulent large-plaque virus could be isolated readily from spleens of moribund animals.

Virus from plaques that grew to diameters of approximately 2 mm was, on occasion, difficult to evaluate on the basis of plaque size alone. Virus from some 2-mm plaques, found in attenuated virus material during experiments, formed only small plaques upon passage and was attenuated for mice. In other instances, virus from 2-mm plaques produced plaques of equal or larger size, containing virulent virus. Whether our encounters with plaques of approximately 2 mm in size resulted from the temporary alteration of environmental conditions that affected only plaque size or whether these plaques contained, in all cases, unstable intermediate-plaque virus is not presently known.

Other investigators (18, 24; Boyle, *personal communication*) have reported intermediate-plaque virus among viral populations. Unpublished data obtained in our laboratory and a number of other reports (15, 22) reviewed recently by Barron and Karzon (1), and by Campbell and Colter (4), have shown that inhibitors in the agar influence the growth and plaque-forming ability of certain particles within a population. One explanation of this was shown by Colon et al. (5), using several group A arboviruses, to be an interaction between an agar polysaccharide and susceptible virus particles; the 9t virus was agar polysaccharide-sensitive, but PES virus was not susceptible. More recently, it was reported that PES virus plaque size also is reduced under agar containing a low bicarbonate concentration that presumably allows the expression of agar inhibition (21). The influence of the virus-inhibiting factor in agar may vary despite precautions, and could impose a degree of uncertainty in immediately recognizing the genuine intermediate-size plaques. Evidence for the selection of intermediate-plaque virus from large-plaque preparations and results of additional work on the recognition and characterization of intermediate-plaque-forming virus are presented elsewhere (11).

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